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Regulation of the ecdysteroid titer of *Manduca sexta*: Reappraisal of the role of the prothoracic glands

(molting hormone/insect development/ketoreductase/2- and 3-dehydroecdysone/ecdysone)

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ABSTRACT **It is generally accepted that the prothoracic glands of insects produce ecdysone, which is converted by a 20-monooxygenase in peripheral tissues to the major molting hormone, 20-hydroxyecdysone. Incubation *in vitro* of the prothoracic glands of larval or pupal *Manduca sexta* in the presence of a hemolymph protein fraction (HPF) increased the ecdysteroid content of the medium almost 8-fold. A comparable increase was noted when HPF was added to medium preconditioned with prothoracic glands but from which the glands had been removed. We used a differential RIA to show that a major product of the prothoracic glands *in vitro* cross-reacts with antiserum (20-hydroxyecdysone-2-succinylthyroglobulin amide; H-2) that retains affinity to ecdysteroids having a modified A ring. However, this product did not bind to antiserum (ecdysone-22-succinylthyroglobulin amide; H-22) that has affinity mainly for ecdysteroids modified at the side chain. We employed radiolabeled precursor studies with prothoracic glands *in vitro* and a combination of analytical techniques (NMR, CD, MS) to demonstrate that the major ecdysteroid released from the glands is a mixture of 2-dehydroecdysone and 3-dehydroecdysone (1:2), which is rapidly reduced to ecdysone in the presence of HPF. We postulate that the active component of HPF is 3β(2β)-forming-3(2)-ketoecdysteroid reductase. These results may explain several anomalous observations pertaining to the molting of insect fragments in the absence of prothoracic glands and suggest a complex system for the control of insect molting and metamorphosis.**

It has been more than four decades since it was demonstrated that the prothoracic glands play a critical role in insect molting (1) and 33 years since ecdysone, the presumed molting hormone, was crystallized from extracts of 500 kg of *Bombyx mori* pupae (2). Thirteen years ago the relationship between ecdysone and the prothoracic glands was apparently clarified when it was reported that ecdysone was the principal product of the prothoracic glands of *Manduca sexta* (3) and *B. mori* larvae (4). In the intervening years, a central dogma of insect endocrinology has emerged—i.e., a neuropeptide from the insect brain, prothoracicotropic hormone (PTTH), stimulates the prothoracic glands by way of a cyclic nucleotide-protein kinase cascade leading to the synthesis and release of ecdysone (5, 6). Ecdysone is then converted to the principal insect molting hormone, 20-hydroxyecdysone, by an ecdysone 20-monooxygenase in tissues peripheral to the prothoracic glands, and a critical titer of 20-hydroxyecdysone leads to the initiation of the molting process. We report here the existence of a regulatory system that appears to play a critica role in establishing the ecdysteroid titer so crucial for normal insect molting, development, and metamorphosis.

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MATERIALS AND METHODS

Animals. *M. sexta* larvae were reared on an artificial diet at 25–26°C and high humidity (60%) under long-day conditions (light/dark, 16:8) as described (7). *Antheraea polyphemus* pupae were obtained commercially and held at 6°C for 5–9 months.

Hemolymph Fractionation. The antioxidant phenylthiourea was added to hemolymph from fifth instar day 6 *M. sexta* (V_6) larvae or chilled *A. polyphemus* pupae. The hemolymph was fractionated on a Sephadex G-15 (Pharmacia) column to remove low molecular weight moieties, especially ecdysteroids and tyrosine. The residual immunoreactive content of the resulting hemolymph protein fraction (HPF) (<10 ng/ml) was deducted from data resulting from addition of HPF to incubation media.

RIA. The rabbit antisera were obtained following immunization with ecdysone-22-succinylthyroglobulin amide (H-22) or 20-hydroxyecdysone-2-succinylthyroglobulin amide (H-2). RIA using the H-22 antiserum quantifies compounds resulting from side-chain phase I oxidations (carbon atom hydroxylations and/or oxidations of existing hydroxyl groups) and phase II conjugations (esterification of hydroxyl groups with organic or inorganic acids), whereas the H-2 antibodies have similar affinities for molecules resulting from phase I and II metabolism of the A ring (7–11).

Dissection and Culture. Prothoracic glands were dissected from day 0 (P_0) or day 2 (P_2) *M. sexta* pupae. One gland was incubated at 25°C in 50 μl of Grace's medium minus tyrosine and the contralateral gland was incubated in medium containing HPF (1:1). At various times, 20-μl aliquots of medium were assayed for immunoreactive (H-22) ecdysteroids. Twenty microliters of fresh medium (with or without HPF) was added back to the cultures to keep the volumes constant. Alternatively, prothoracic glands (up to 72 pairs) were incubated up to 48 hr in proportionately larger volumes of medium containing streptomycin and gentamycin (each at 100 μg/ml) but without HPF. Duplicate 20-μl aliquots were removed at intervals and replaced with fresh medium. One was assayed for immunoreactive (H-22) ecdysteroids directly, and the other was assayed after treatment with HPF (20 μl, 30 min, 25°C) followed by heat denaturation (100°C, 2

Abbreviations: PTTH, prothoracicotropic hormone; HPF, hemolymph protein fraction; NOE, nuclear Overhauser effect(s); NP, normal-phase; RP, reverse-phase; H-22, ecdysone-22-succinylthyroglobulin amide; H-2, 20-hydroxyecdysone-2-succinylthyroglobulin amide; DCI, desorption chemical ionization.

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min) and centrifugation (5 min, 10,000 × *g*) to remove protein.

Chemicals. Ecdysone (Simes, Milan, Italy) was chemically oxidized (O_2/Pt) and the product(s) was purified by using established procedures (12–14): desorption chemical ionization (DCI)-MS (NH_3) *m/z* 480 (M + NH_4^+), negative DCI-MS (NH_3) *m/e* 461 (M − H). An identical oxidation was performed in ^2H_2O. Tritiated product was obtained by including [^{3}H]ecdysone (60 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear).

Chromatography. Prothoracic gland-conditioned medium or the synthetic oxidation product(s) of ecdysone was applied to primed C_{18} Sep-Paks (Waters, Milford, MA), which were then washed with water, the ecdysteroids were eluted with methanol, the solvents were evaporated under reduced pressure, and the residues were subsequently purified by analytical reverse-phase (RP)-HPLC and normal-phase (NP)-HPLC (where HP = high-performance) or TLC. Aliquots from HPLC or eluted TLC sections were subsequently analyzed for radioactivity or immunoreactivity (7–9).

Spectroscopy. UV spectra were recorded on a Shimadzu UV-3000 spectrophotometer. CD analysis utilized a Jasco J-500A with the sample in methanol. NMR spectra of compounds in ^2H_2O or methanol-d_4 were recorded on a Varian-400XT or Bruker WM-250 with either $^1HO^2H$ (δ4.67) or C^2H_3OH (δ3.30) as reference standard. DCI-MS were taken on a Ribermag 10-10 instrument with ammonia as ion source.

RESULTS

Physiological Observations. To determine if *M. sexta* prothoracic glands were stimulated by hemolymph as were those of *B. mori* (4), prothoracic glands from P_0 pupae were placed in lepidopteran saline, one of the pair acting as control and its contralateral mate receiving HPF from *A. polyphemus*. After 2 hr of incubation, the HPF medium contained 7.8-fold the amount of ecdysteroids as the control, as determined with the H-22 RIA. Indeed, it appeared that ecdysteroid production was accelerated as soon as the prothoracic glands were transferred to medium containing HPF (Fig. 1). Similar results were obtained with glands from V_7 larvae and P_2 pupae. Although the data suggested that the HPF contained a factor that stimulated ecdysone production by the glands, it was also possible that the HPF contained a substrate that could be enzymatically converted to ecdysone by the prothoracic glands or that the HPF contained a factor (enzyme?) that converted a weakly cross-reacting (H-22) ecdysteroid produced by the glands into a strongly cross-reacting ecdysteroid—i.e., ecdysone.

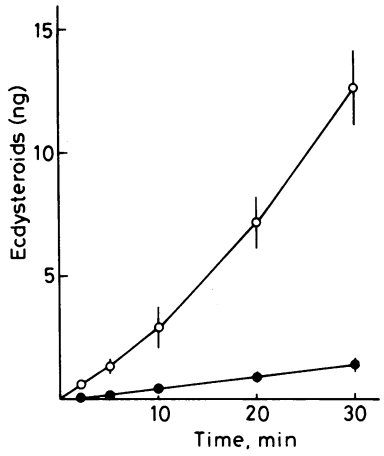

FIG. 1. Time course of immunoreactive (H-22) ecdysteroid production by P_0 prothoracic glands *in vitro*. *M. sexta* prothoracic glands were incubated in lepidopteran saline (•) or saline with HPF (○). Each point is an average of four separate determinations ± SD.

To investigate the above possibilities further, four pairs of P_0 prothoracic glands were incubated in 200 μl of Grace's medium for 30 min, the glands were removed, and HPF was added to an aliquot of this preconditioned medium. Measured by the H-22 RIA, the preconditioned medium contained 1.79 ng of immunoreactive ecdysteroid per gland *before* addition of HPF, and 12.97 ng *after* the addition of HPF (a 7.25-fold increase in the *absence* of glandular material), thus excluding the possibility that HPF stimulated the gland directly. Next, the HPF was boiled for 2 min and centrifuged to remove denatured protein, and the supernatant was tested on the preconditioned medium. The heat-treated material was completely ineffective, suggesting the presence in the HPF of a heat-labile moiety (enzyme?) that was responsible for the observed increases in H-22 immunoreactive ecdysteroids in the medium. Prior heating of the prothoracic gland preconditioned medium had no effect on the HPF-mediated increase in immunoreactive ecdysteroids. Butanol extraction of preconditioned medium yielded 20.40 ng of H-22 immunoreactive ecdysteroids (the original medium contained 25.46 ng), whereas the solvent residue after 30 min of treatment with HPF contained 108.00 ng, a 5.29-fold increase.

These observations indicate that the prothoracic glands produce a heat-stable ecdysteroid (X) not recognized by the H-22 antiserum but which is extractable with butanol and is converted to an immunoreactive ecdysteroid by a heat-labile enzyme found in the hemolymph of *A. polyphemus*. The factor (enzyme) was also found in the hemolymph of larval and pupal *M. sexta* (S.S., J.T.W., D.B.R., and L.I.G., unpublished data). The prothoracic glands from several other species of Lepidoptera also secreted X, and all of these species also yielded hemolymph capable of converting X to ecdysone (D.B.R., J.T.W., S.S., and L.I.G., unpublished data).

The kinetics of ecdysone and X production by prothoracic glands *in vitro* are shown in Fig. 2. Glands (P_2, 18 pairs) were incubated in Grace's medium minus tyrosine (1 ml) and aliquots were analyzed for H-22 immunoreactive ecdysteroids prior to and following HPF (from *M. sexta* pupae) treatment. Since ecdysone and X comprise >99% of the ecdysteroids in the medium, the assay measures the cumulative production of ecdysone or, after HPF treatment, the cumulative production of ecdysone plus X (in ecdysone equivalents). The difference represents the amount of X in the medium. From the beginning of incubation, X is produced rapidly, achieving a concentration 7-fold that of ecdysone after 1 hr and a maximum at 6 hr, about the time when production of total ecdysteroids by the glands approaches zero. Subsequently, the concentration of X declines at a rate of 0.78 ng per gland per hr, the same rate at which ecdysone accumulates in the medium during this period. The rate of ecdysone production remains relatively constant throughout the incubation period, even after the glands no longer secrete additional ecdysteroid. It is apparent that the primary, if not sole, secretory product of the glands is X, which is slowly converted to ecdysone so after long-term incubation, ecdysone is the major ecdysteroid isolated from the medium. Addition of HPF from a variety of developmental stages greatly accelerates this conversion.

Concurrent studies on the biosynthesis of ecdysone by *M. sexta* prothoracic glands revealed that [^{3}H]- or [^{14}C]cholesterol was converted into labeled 7-dehydrocholesterol in the glands. In the medium, label was ultimately detected in ecdysone and in a less polar ecdysteroid that cross-reacted strongly with the H-2 antibody, but did not cross-react with the H-22 antibody (9). These data indicate that the unidentified ecdysteroid was modified in the A ring rather than in

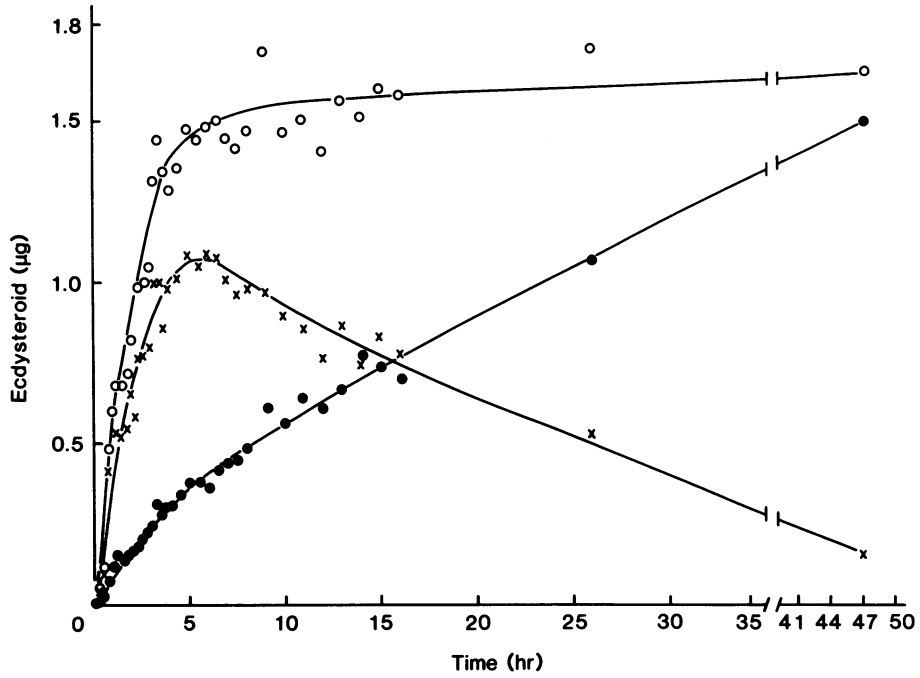

FIG. 2. Time course of cumulative ecdysone and X production by *M. sexta* prothoracic glands (P_2) incubated in Grace's medium minus tyrosine. At each time point an aliquot of medium was analyzed by RIA (H-22) prior to (●) or after incubation (30 min) with HPF (○). The difference represents the medium content of X (x) in ecdysone equivalents.

the side chain (see *Materials and Methods*). To determine if X was identical to the aforementioned less polar ecdysteroid, 24 pairs of P_0 prothoracic glands were incubated in 0.7 ml of Grace's medium for 1 hr. The medium then was analyzed by RP-HPLC. An aliquot (20 μl) of each fraction from the HPLC was subjected to RIA with the H-22 and H-2 antisera. In addition, a separate aliquot (10 μl) after solvent removal was treated with HPF (20 μl in 20 μl of Grace's medium minus tyrosine) and subjected to H-2 and H-22 RIA. Fig. 3 reveals that ecdysone eluted between fractions 34 and 38, and the quantity of ecdysone present was the same as measured with each antibody and was not affected by the addition of *M. sexta* HPF (compare Fig. 3 *A* and *B*). In the absence of HPF an H-2 immunoreactive peak was noted between fractions 42 and 48 (Fig. 3*A*), which was not apparent when the H-22 RIA was utilized (Fig. 3*B*). After the addition of HPF, the total H-22-positive ecdysteroid in this second peak (X) increased from 36.2 ng to 773.0 ng (21.4-fold), whereas with the H-2 antibody, the ecdysteroid content of the X peak increased only 2.2-fold. These numbers, 21.4 and 2.2, represent the cross-reactivities of X relative to ecdysone with the H-22 and H-2 antisera, respectively. After HPF treatment of the X peak and subsequent RP-HPLC and NP-HPLC, immunoreactive material using either the H-22 or H-2 antiserum was found only at the retention time of ecdysone. Further analysis (RP-HPLC and NP-HPLC) indicated that X was identical to the compound observed when glands were incubated with labeled cholesterol or 7-dehydrocholesterol. In addition, when [^{3}H]X of biosynthetic origin (1 μg, 20 mCi/mmol) was treated for 2 hr at room temperature with HPF (100 μl in 100 μl of Grace's medium minus tyrosine) and the product was analyzed by RP-HPLC, NP-HPLC, and HP-TLC, conversion to [^{3}H]ecdysone was quantitative. The synthetic [^{3}H]ecdysone oxidation product(s) (1 μg, 6 mCi/mmol) were also completely converted to [^{3}H]ecdysone by HPF. Neither ecdysone nor X was present in extracts of the prothoracic glands prior to or following their *in vitro* incubation.

The presence of this previously unrecognized ecdysteroid in medium in which prothoracic glands had been incubated was established by four criteria: (*i*) detection with the H-2 antiserum; (*ii*) conversion of [^{3}H]cholesterol into X; (*iii*) conversion of 7-dehydro[^{3}H]cholesterol into X; and (*iv*) absorbance of the X peak at 242 nm. We next turned our attention to the identification of X.

Chemical Characterization of X. The identical UV data for ecdysone (I) (Fig. 4) and compound X (λ_{max} = 243 nm, ε = 12,400) showed the enone moiety to be intact. The following data show that X is an ≈1:2 mixture of 2-dehydroecdysone (II), and 3-dehydroecdysone (III). A comparison of the CD spectra of compound X and ecdysone (I) indicated the presence of an extra saturated carbonyl function—namely, the CD of I shows Cotton effects (CE) at 330 nm ($\Delta\varepsilon$+2.3, $\eta\pi^*$), 250 nm ($\Delta\varepsilon$−5.2, $\pi\pi^*$) (15), and 223 nm ($\Delta\varepsilon$+3.4), whereas that of X shows CE at 335 nm, 227 nm ($\eta\pi^*$ of extra ketone), 257 nm, and 222 nm. The molecular ion of the mass spectrum of X corresponded to the oxidation of one sec-OH to a carbonyl. From the antibody cross-reactivity data, it was likely that this oxidation had occurred in the A ring rather than the side chain.

The ^{1}H NMR spectrum of X (Fig. 5) showed the presence of two 7-H signals at 5.90 and 5.85 ppm (ratio 2:1) and twin peaks due to 22-H (3.55) and 9-H (3.30/3.00); this together with the difference in chemical shifts of 18-H (δ0.59 for I, δ0.61 for major component in X) and 19-H (δ0.86 for I, 0.90 for major component in X) suggested that X could be a mixture of 2- and 3-dehydroecdysone. This was proven by oxidation of ecdysone with O_2/Pt to products, the CD and NMR data (Fig. 5)[‖] of which were very similar to those of mixture X. 3-Dehydroecdysone (III) had been reported to be the sole oxidation product of ecdysone (12–14), but it was found that II, as well as III, is formed by varying the amount of catalyst. The ratio of II to III depends on the catalyst/ecdysone ratio; the II/III ratio approximates that in compound X when near equal amounts of Pt and ecdysone are employed.

That the major oxidation product is, in fact, 3-dehydroecdysone (III) is corroborated by measurements of NOE (Fig. 4), which showed that irradiation of 9-H (δ3.30, in III) enhanced the signals of 2-H (δ4.60, *dd*, J = 12 and 5 Hz) and 4-H_{ax} (δ2.71, *t*, J = 13.5), a typical result encountered in

[‖]Chemical shifts of pertinent NMR peaks of II ("a") and III ("b"): 26/27-H (Me), all at δ1.06; 21-H (Me), δ0.77 *d* in a and δ0.79 *d* in b; 18-H (Me), δ0.58 *s* in a and δ0.61 *s* in b; 7-H, δ5.85 *d* in a and δ5.90 *d* in b; 19-H (Me), δ0.81 *s* in a and δ0.90 *s* in b; 9-H, δ3.00 *m* in a and δ3.30 *m* in b.

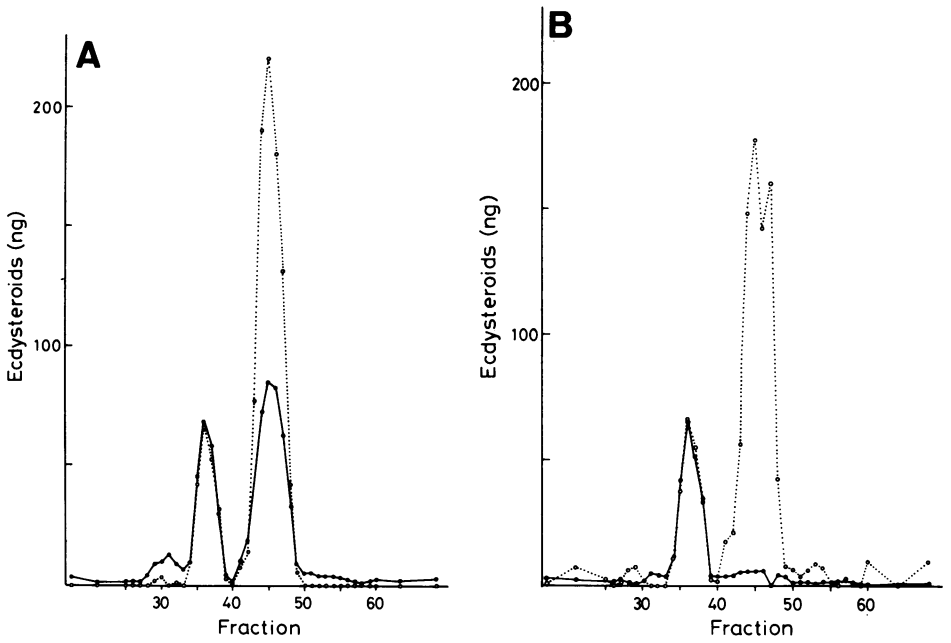

FIG. 3. RP-HPLC/RIA analysis of ecdysteroids in medium after *in vitro* incubation of *M. sexta* P_0 prothoracic glands for 1 hr at 25°C. RP-HPLC utilized a Resolve C_{18} (5 μm, Waters) column and a solvent system of acetonitrile/20 mM Tris/perchlorate, pH 7.5; 5–95% acetonitrile in 60 min (1 ml/min). (*A*) Analysis by H-2 antiserum of aliquots of elution fractions prior to (—●—) and after incubation with HPF (···○···). (*B*) Analysis by H-22 antiserum of corresponding aliquots of identical fractions as in *A* prior to (—●—) and after incubation with HPF (···○···). Fractions 34–38 contain ecdysone; fractions 42–48 contain X. After treatment with HPF, the X in fractions 42–48 has been reduced to ecdysone. The increase in immunoreactivity of these fractions after HPF addition is due to the greater affinity of both antibody preparations for ecdysone relative to X.

ecdysteroids. In contrast, II shows a signal at 3.85 ppm (*dd*, J = 12 and 4 Hz, 3-H), the intensity of which is enhanced upon irradiation of 9-H (coupled to 7-H at δ5.85); a NOE is also observed between 9-H and 12-H_{ax} (δ1.54). Molecular models indicate that ring A in II must adopt a twist boat conformation to account for those NOE results.

Products II and III show that the platinum-catalyzed reaction is a straightforward oxidation. This was corroborated by carrying out the oxidation in ^2H_2O upon which no deuterium was incorporated at C-2, -3, -4, and -5—namely, the production of II and III does not involve enol intermediates. Despite intensive efforts, it has not been possible to achieve a baseline separation of these two compounds by NP or RP chromatography.

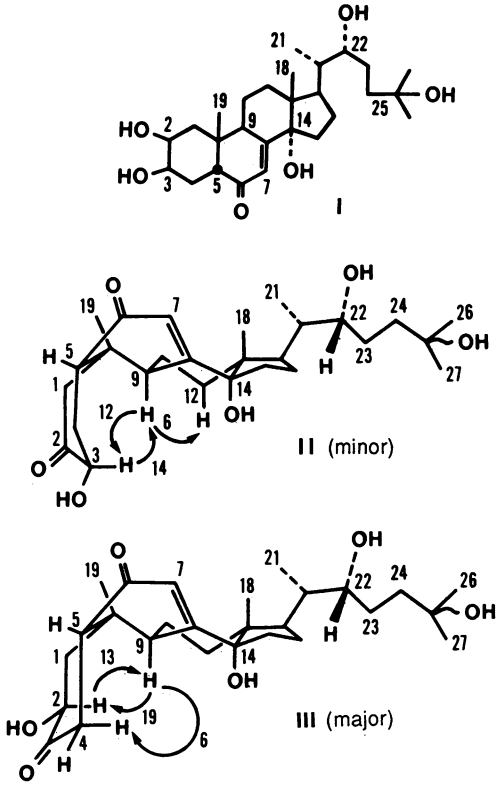

FIG. 4. Ecdysone (I), 2-dehydroecdysone (II), and 3-dehydroecdysone (III). NMR nuclear Overhauser effects (NOE) (in methanol-d_4) are shown in conformational structures II and III; the number by each arrow denotes the relative % increment of peak intensities accompanying irradiation.

DISCUSSION

The data presented here indicate that the prothoracic glands synthesize and secrete 3-dehydroecdysone and 2-dehydroecdysone (2:1) and a much smaller amount of ecdysone, or perhaps no ecdysone at all. Further, 2- and 3-dehydroecdysone are converted to ecdysone by a factor in the hemolymph, most likely a 3β(2β)-forming-3(2)-ketoecdysteroid reductase. The analytical chemical data based on NMR, MS analysis, and CD studies leave little doubt regarding the nature of the ecdysteroids recovered from the medium in which prothoracic glands have been incubated.

When the original studies were conducted (3, 4), the prothoracic glands were mass cultured for up to 15 days to obtain as much product as possible for chemical identification, without making the effort necessary to remove adhering hemolymph. The present data reveal that even under the best conditions, most of the secreted 2- and 3-dehydroecdysone is converted to ecdysone within 30 min in the presence of added hemolymph or after 48 hr in prothoracic gland culture alone. In fact, the possibility exists that the prothoracic glands actually synthesize no ecdysone at all, but that the basal lamina (sheath) forming the outer boundary of the glands, or material (e.g., hemocytes, enzyme) adhering to this noncellular sheath, have the capacity to reduce 2- and 3-dehydroecdysone to ecdysone. By using an enzymatic digestion procedure, one can obtain dissociated prothoracic gland cells devoid of the sheath that respond normally to PTTH by enhanced ecdysteroid synthesis (16). When a similar paradigm was utilized to investigate the production of 2- and 3-dehydroecdysone, it was revealed that well-washed, dissociated prothoracic gland cells without basal lamina produce a much greater ratio of 2- and 3-dehydroecdysone/ecdysone than intact glands (D.B.R., J.T.W., S.S., and L.I.G., unpublished data). In the 1970s, we had just begun to understand the diversity of ecdysteroids in insect tissues (see ref. 17) and believed that the prothoracic glands must synthesize and secrete ecdysone and/or 20-hydroxyecdy-

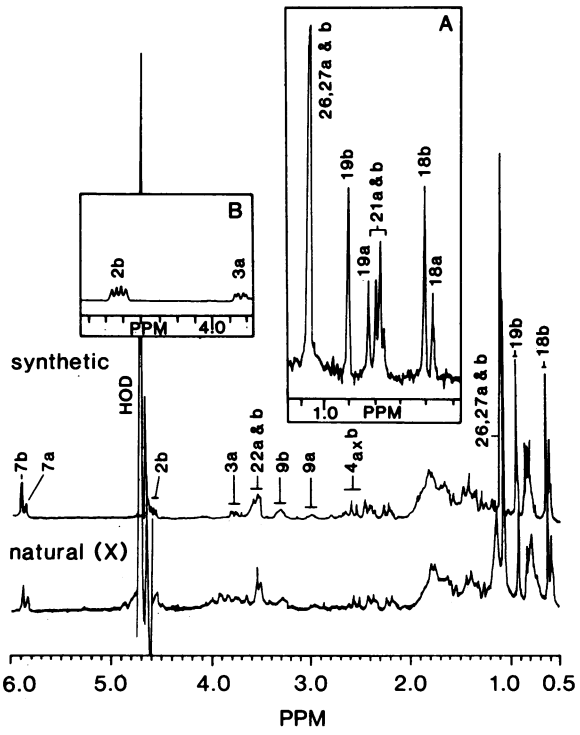

FIG. 5. NMR spectra of compound X and synthetic oxidation products of ecdysone, 250 MHz, in ^2H_2O. (*Inset A*) Detail of compound X methyl peaks, 400 MHz, in ^2H_2O. (*Inset B*) Synthetic product ring A peaks, 250 MHz, in methanol-d_4. Compound X and the synthetic product are ≈ 1:2 mixtures of 2-dehydroecdysone (II) and 3-dehydroecdysone (III); peaks belonging to II and III are designated by postscripts "a" and "b," respectively. HOD, $^1HO^2H$.

sone. This may have led to the discounting of "spurious" peaks in the several chromatographic procedures utilized. On the other hand, a number of past observations support the present concept that a preecdysone molecule is synthesized by the prothoracic glands. There have been a variety of observations indicating that insects devoid of prothoracic glands can molt (18). Perhaps 2- and 3-dehydroecdysone are sequestered by tissues when the hemolymph ketoreductase titer is low and manipulations such as surgery release stored oxoecdysteroids from the sequestration compartment in one or more tissues into the hemolymph, where they are converted to ecdysone and then to 20-hydroxyecdysone. The conversion of a prohormone to more active forms by reductase activity is not unique to insects since, for example, it is well known that the conversion of testosterone to dihydrotestosterone is mediated by a 5α-reductase, and the latter compound is converted to 5α-androstanediol in the presence of 3-ketoreductase (19). Further it is possible that 3-dehydroecdysone has hormonal activity itself since it elicits an ecdysteroid-specific puffing pattern from *Drosophila melanogaster* salivary gland polytene chromosome (20), albeit at high concentrations, and just recently it has been reported that 3-dehydro-20-hydroxyecdysone is an order of magnitude more effective than 20-hydroxyecdysone in stimulating specific protein synthesis in *D. melanogaster* fat body *in vitro* (21).

The data reveal that under *in vitro* conditions the prothoracic glands of *M. sexta* synthesize and secrete 2- and 3-dehydroecdysone as major products. One could argue that this is a result of the oxidation of ecdysone to 3-dehydroecdysone by an ecdysone oxidase (22, 23). However, incubation of [^{3}H]ecdysone with prothoracic glands, homogenates of prothoracic glands, or dialyzed cytosol from prothoracic glands under oxidizing conditions yields insignificant quantities of 2- or 3-dehydro[^{3}H]ecdysone (J.T.W. and L.I.G., unpublished data). It must be emphasized that our studies have been performed *in vitro* and although there is no doubt that *M. sexta* prothoracic glands synthesize 3-dehydroecdysone and 2-dehydroecdysone by way of cholesterol and 7-dehydrocholesterol (9), it has *not* been demonstrated that these ecdysteroids are released from the prothoracic glands *in situ*. Since a putative (2)3-ketoreductase, or at least a factor that mediates the conversion of 2- and 3-dehydroecdysone to ecdysone, exists in the hemolymph, any of the former compounds would be rapidly reduced to ecdysone, or perhaps sequestered by peripheral tissues and are, therefore, not detectable in an analysis of the hemolymph. Indeed, when [^{3}H]X (2 μg) was injected into *M. sexta* pupae and the hemolymph was sampled 10 min later, only ecdysone and ecdysone metabolites (26-hydroxyecdysone and highly polar conjugates) were identified by RP-HPLC and NP-HPLC (J.T.W. and L.I.G., unpublished data).

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